REMARKS

Claims 1-8 are pending in the instant application. Claims 1-4 have been withdrawn from consideration by the Examiner and subsequently canceled by Applicants, without prejudice, by this amendment. Claims 5-8 have been rejected. Claims 5 and 6 have been amended. Claims 7 and 8 have been canceled in light of the amendments to claim 5. No new matter is added by these amendments. Reconsideration is respectfully requested in light of these amendments and the following remarks.

I.   Finality of Restriction Requirement

The Examiner has made final the Restriction Requirement mailed October 8, 2002. Accordingly, in an earnest effort to advance the prosecution of this case, Applicants have canceled nonelected claims 1-4, without prejudice. In light of the finality of the Restriction Requirement, Applicants reserve the right to file a divisional application to the canceled subject matter.

II.   Priority Claim

In accordance with the Examiner's suggestion, Applicants have amended the specification at page 1 to include reference to prior applications, namely the provisional and PCT application,

from which the instant application claims priority. Withdrawal

of this object to the specification is therefore respectfully

requested.

III. Rejection of Claims 5-8 under 35 U.S.C. § 112, first

paragraph - Written Description

Claims 5-8 have been rejected under 35 U.S.C. § 112, first

paragraph, as containing subject matter which was not described

in the specification in such a way as to reasonably convey to one

skilled in the relevant art that the inventors, at the time

application was filed, had possession of the claimed invention.

The Examiner suggests that a generic statement such as "non-

internalizable antibody" is not an adequate written description

of the genus because it does not distinguish the claimed genus

from others, except by the property of being non-internalizable.

The Examiner suggests that this phrase does not specifically

define any of the proteins that fall within its definition and

does not define any structural features commonly possessed by

members of the genus that distinguish them from others.

Applicants respectfully traverse this rejection.

MPEP § 2163 and the case law are clear; an adequate written

description of the invention may be shown by any description of

sufficient, relevant, identifying characteristics so long as a

person skilled in the art would recognize that the inventor had possession of the claimed invention. See e.g. Purdue Pharma LPV v. Faulding, Inc. 230 F3d 1320,1323, 56 USPQ2d 1481, 1483 (Fed. Cir. 2000). Although structural formulas provide a convenient method of demonstrating possession of specific molecules, other identifying characteristics or combination of characteristics may demonstrate the requisite possession. See MPEP § 2163.

At page 9, lines 9-20 of the specification, non-internalizable antibodies are defined as antibodies which bind to an antigen on the luminal surface of the pulmonary vasculature and which are determined not to be internalized by cultured human endothelial cells as described in the application and/or is shown to be temperature independent in pulmonary uptake experiments in isolated lung perfusions as also described. Accordingly, both the structure of this genus of molecules, an antibody, and a functional characteristic of this genus of molecules, binding to an antigen on the luminal surface of the pulmonary vasculature, which is not internalized, are taught by the written description of the application. Therefore, the written description requirement for the claimed genus of non-internalizable antibodies is satisfied by this disclosure. See MPEP § 2163.

In an earnest effort to advance the prosecution of this case, claim 5 has been amended to clarify properties and/or identifying characteristics of the antibodies used in the claimed method, which distinguish them from other antibodies. Specifically, claim 5 has been amended to delete the term "non-internalizable" and to state instead that the antibody binds to an antigen on the luminal surface of the pulmonary vascular endothelium without subsequent internalization into endothelial cells. Support for this amendment is provided in the specification at page 9, lines 9-20 wherein methods for identifying antibodies with these characteristics are set forth as well. Clearly, one of skill in the art, upon reading the instant specification, would recognize that the inventor had possession of the genus of antibodies used in the method as set forth in the amended claims. Thus, the instant specification meets the written description requirements for the invention as claimed.

Withdrawal of this rejection under 35, U.S.C. § 112, first paragraph for written description, is therefore respectfully requested.

IV.   Rejection of Claims 5-8 under 35 U.S.C. § 112, first paragraph - Lack of Enablement

Claims 5-8 have also been rejected under 35 U.S.C. § 112, first paragraph, for lack of enablement. The Examiner has acknowledged the specification to be enabling for dissolution of fibrin clots by administering a non-internalizable antibody to ICAM-1 and a fibrinolytic or anti-coagulant. However, the Examiner suggests that the specification does not reasonably provide enablement for the claimed method of treatment *in vivo* using a non-internalizable antibody that is not an anti-ICAM-1, nor the claimed method of prevention using any non-internalizable antibody, including anti-ICAM-1.

Applicants respectfully traverse this rejection.

The analysis of whether a particular claim is supported by the disclosure of an application requires a determination of whether that disclosure, when filed, contained sufficient information regarding the subject matter of the claims as to enable one skilled in the art to make and use the claimed invention. MPEP § 2164.01.

With respect to using a non-internalizable antibody other than anti-ICAM-1, Applicants have provided explicit details in the specification so that one of skill in the art can identify

routinely additional non-internalizable antibodies useful in the

claimed method of the present invention. The Examiner is

respectfully directed to page 9, lines 9-20 of the specification

wherein it is taught that a non-internalizable antibody useful in

the present invention can be identified by determining its

ability to bind to an antigen on the luminal surface of the

pulmonary vasculature, its ability not to be internalized by

cultured human endothelial cells (methods of which are set forth

in Example 2 of the specification) and/or its temperature

independence in pulmonary uptake experiments in isolated lung

perfusions (methods of which are set forth in Example 3 of the

specification). Such methods can be performed routinely by those

skill in the art and do not constitute undue experimentation.

Thus, the teachings of the specification clearly enable one of

skill in the art to make and use other non-internalizable

antibodies in the invention commensurate in scope with the

claims.

Further, Applicants are providing herewith a publication by

Murciano et al. (Am. J. Crit. Care, October 1, 2001 163:1295-

1302) demonstrating use of a different antibody, which binds not

to ICAM-1, but to a distinct antigen (GP85) on the luminal

surface of the vascular endothelium without subsequent

internalization into endothelial cells in vascular

immunotargeting. This reference, which was published by the

inventor subsequent to the filing date of the instant

application, used methods taught in the instant application to

identify antibody mAb 30B3 as an antibody which binds to an

antigen on the luminal surface of the vascular endothelium

without subsequent internalization into endothelial cells. See

specifically page 1296 of this reference wherein experiments

involving perfusion of the isolated rat lung (as described in

Example 5 of the instant application) with this different

antibody are described. Also see page 1296 wherein in vivo

administration of radiolabeled antibodies (as described in

Example 6 of the instant application) with this different

antibody are described. This reference also demonstrates yet

another method for documenting lack of antibodies internalization

in the pulmonary endothelium, namely immuno-gold Transmission

Electron Microscopy (TEM) of the lung tissue sections (see Figure

8 of the publication). A similar technique was used in another

recent publication by the inventors, Murciano et al. (Blood, 2003

MS 2002-09-2853), a copy of which is provided herewith, to

confirm that anti-ICAM-1 also accumulated in the pulmonary

vasculature after injection in mice and did not undergo

internalization (Fig.5). These references clearly evidence the ability of one of skill in the art to make and use other non-internalizable antibodies beside anti-ICAM-1 in the invention commensurate in scope with the claims.

Further, Applicants respectfully disagree with the Examiner's suggestion that the instant application is not enabling for a method of preventing intravascular coagulation. The ability of the claimed method to dissolve fibrin clots, which has been acknowledged to be enabled by the Examiner, by its very nature prevents intravascular thrombosis.

The ability of the claimed method to augment the local anti-thrombotic potential of endothelium and dissolve intravascular blood clots has also been confirmed in animals. Results from these experiments were recently published online in Blood MS 2002-09-2853 (discussed above). A copy of this online publication is provided herewith. The Examiner is respectfully directed to page 24-25 and Figure 9 of this paper wherein it is demonstrated that enzymatically active anti-ICAM/tPA conjugate accumulated in the pulmonary vasculature and facilitated fibrinolysis of fibrin emboli lodged in rat lungs.

In an earnest effort to advance the prosecution of this case, Applicants have amended claim 5 to remove the phrase

"prevention of intravascular coagulation". Further, in accordance with the statement at page 8, lines 32-35, and the experiments described at page 8, line 15-35, of the specification supporting this statement, Applicants have amended claim 1 to include in the preamble that the method also augments local anti-thrombotic potential of endothelium.

Withdrawal of this rejection under 35 U.S.C. § 112, first paragraph, is respectfully requested in light of the amendments to the claims and the above arguments.

V.   Rejection of Claims 5-8 under 35 U.S.C. § 103(a)

Claims 5 and 6 have been rejected under 35 U.S.C. § 103(a) as being unpatentable over Bowes et al. (Neurology 1995) in view of Imaizumi (Transpl. Proc. 1994) and further in view of Mulligan et al. (Amer. J. Pathol. 1993) and Panes (Amer. J. Physiol. 1995).

Claims 7 and 8 have also been rejected under 35 U.S.C. § 103(a) as being unpatentable over Bowes et al. (Neurology 1995) in view of Imaizumi (Transpl. Proc. 1994) and further in view of Mulligan et al. (Amer. J. Pathol. 1993) and Panes (Amer. J. Physiol. 1995) as applied above, and further in view of Torchilin et al. (J. Contr. Rel. 1985), Muzykantov et al. (BBA 1986) and Muzykantov et al. (Amer. J. Physiol. 1996).

Applicants respectfully traverse these rejections.

At the outset, it is respectfully pointed out that claim 5 has been amended to include the limitation of claim 7. Claims 7 and 8 have been canceled in light of the amendment to claim 5. Accordingly, claim 5 is now drawn to a method of augmenting local anti-thrombotic potential of endothelium and dissolving intravascular clots in the pulmonary vasculature of an animal comprising administering to the animal a fibrinolytic or anticoagulant agent **conjugated with an** antibody which binds to an antigen on the luminal surface of the vascular endothelium without subsequent internalization into endothelial cells.

Neither the combination of cited references of Bowes et al. (Neurology 1995) in view of Imaizumi (Transpl. Proc. 1994) and further in view of Mulligan et al. (Amer. J. Pathol. 1993) and Panes (Amer. J. Physiol. 1995) nor Bowes et al. (Neurology 1995) in view of Imaizumi (Transpl. Proc. 1994) and further in view of Mulligan et al. (Amer. J. Pathol. 1993) and Panes (Amer. J. Physiol. 1995) as applied above, and further in view of Torchilin et al. (J. Contr. Rel. 1985), Muzykantov et al. (BBA 1986) and Muzykantov et al. (Amer. J. Physiol. 1996). establish a *prima facie* case of obviousness against claim 5 as amended nor claim 6 which is dependent therefrom since neither of

the cited combinations of references teach or suggest all the limitation of the claims. In particular, none of the cited references teach or suggest conjugation of the therapeutic agent to a non-internalizable antibody.

In each of the cited prior art references, the effects of a fibrinolytic agent were examined alone and when co-administered with an antibody. Nowhere are the effects of conjugating a thrombolytic agent with an antibody targeted to a poorly internalized antigen of the endothelium taught or suggested.

Instead, Bowes et al. (Neurology 1995) teach administration of a anti-ICAM-1 antibody in combination with tPA in an animal model of cerebral embolism stroke for study of the thrombolytic effects of tPA both alone and in the presence of the antibody. The results showed that the combination of the two did not work better than each compound alone in reducing neurological damage. Nowhere does this paper teach or suggest targeting the luminal surface of pulmonary vascular endothelium with a drug conjugated to a non-internalizable antibody.

Imaizumi (1994) discloses that use of an antibody to ICAM-1, 1A29, significantly enhanced the inhibitory effect of an anti-adhesion molecule antibody, WT.1, on adhesion of neutrophils to vascular endothelial cells. Nowhere does this paper teach or

suggest targeting the luminal surface of pulmonary vascular endothelium with a drug conjugated to a non-internalizable antibody.

Panes et al. (1995) disclose that the 1A29 anti-ICAM-1 antibody reacts with normal endothelial cells in the rat vasculature and that injection of TNF or endotoxin stimulates endothelial binding of this antibody. Nowhere, however, does this paper teach or suggest targeting the luminal surface of pulmonary vascular endothelium with a drug conjugated to a non-internalizable antibody.

Mulligan et al. (1993) teaches that radiolabelled mAb 1A29 accumulates in the vasculature challenged with pro-inflammatory agents such as TNF and endotoxin. However, nowhere does this paper teach or suggest targeting the luminal surface of pulmonary vascular endothelium with a drug conjugated to a non-internalizable antibody.

Torchilin and Mazeav (1985) teaches that enzymes such as tPA can be made more effective by administration in slow release preparations as well as by modifying the enzyme with a compound that has the ability to bind to a blood clot. There is a general discussion of using antibodies as one such modification tool. Nowhere, however, does this prior art reference teach or suggest

administration of a drug in combination with a non-internalizable antibody that binds to an antigen on the luminal surface of the pulmonary vascular endothelium. Further, targeting of a blood clot cannot be used prophylactically to augment local anti-thrombotic potential of endothelium and dissolve intravascular blood clots as claimed since the target has not yet formed. In addition, effectiveness of the agents targeting the blood clot itself such as those taught by Torchilin and Mazaev have been found to be generally restricted because of the inability to penetrate into the clot wherein fibrinolysis is desired.

Muzykantov et al. (1986) discloses an anti-collagen antibody-erythrocyte-streptokinase complex that is formed using an avidin-biotin interaction. This complex has been designed to deliver fibrinolytics to the extravascular interstitium, which is inaccessible for blood under normal and most pathological conditions (except rare conditions associated with overt disruption of the integrity of a blood vessel). Nowhere does this paper teach or suggest administration of a drug conjugated to a non-internalizable antibody which binds to an antigen normally expressed on the luminal surface of the pulmonary vascular endothelium nor augmentation of the local anti-thrombotic potential of endothelium.

Muzykantov et al. (1996) teaches that endothelial cells internalize antibodies against angiotensin-converting enzyme and that this provides for a useful method of targeting genes by intracellular delivery of the agent conjugated to such an antibody. This paper does not teach administration of a drug conjugated to a non-internalizable antibody which binds to an antigen on the luminal surface of the pulmonary vascular endothelium.

Therefore, the cited combinations of prior art cannot render obvious claim 5, as amended, and claim 6, which depends therefrom. Withdrawal of these rejections under 35 U.S.C. § 103(a) is therefore respectfully requested.

VI. Conclusion

Applicants believe that the foregoing comprises a full and complete response to the Office Action of record. Accordingly, favorable reconsideration and subsequent allowance of the pending claims is earnestly solicited.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Claims:

Claims 1-4 and 7-8 have been canceled without prejudice.

Claims 5 and 6 have been amended as follows:
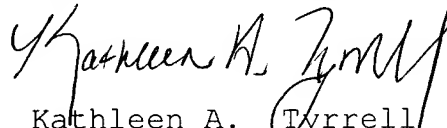
5.   (amended) A method for <u>augmenting local anti-thrombotic</u> <u>potential of endothelium and dissolving of intravascular blood</u> <u>clots</u> in the pulmonary vasculature of an animal comprising <u>intravenously</u> administering to the animal a fibrinolytic or anticoagulant <u>agent conjugated with an</u> antibody which binds to an antigen on the luminal surface of the vascular endothelium <u>without subsequent internalization into endothelial cells</u>.

6.   (amended) The method of claim 5 wherein the ~~non-internalizable~~ antibody is an anti-ICAM-1 antibody.

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Inventors: **Muzykantov et al.**
Serial No.:   **09/762,023**
Filing Date: **June 28, 2001**
Page 17

attached page is captioned **"VERSION WITH MARKINGS TO SHOW CHANGES**

MADE."

                              Respectfully submitted,

                              Kathleen A. Tyrrell
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ICAM-DIRECTED VASCULAR IMMUNOTARGETING OF ANTI-THROMBOTIC AGENTS TO THE ENDOTHELIAL LUMINAL SURFACE

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ABSTRACT

Drug targeting to a highly expressed, non-internalizable determinant up-regulated on the perturbed endothelium may help to manage inflammation and thrombosis. We tested whether Inter-Cellular Adhesion Molecule-1 (ICAM-1) targeting is suitable to deliver anti-thrombotic drugs to the pulmonary vascular lumen. ICAM-1 antibodies bind to the surface of endothelial cells in culture, perfused lungs and *in vivo*. Pro-inflammatory cytokines enhance anti-ICAM binding to the endothelium without inducing internalization. ^{125}I-labeled anti-ICAM and a reporter enzyme (β-Gal) conjugated to anti-ICAM bind to endothelium and accumulate in the lungs after intravenous administration in rats and mice. Anti-ICAM is seen to localize predominantly on the luminal surface of the pulmonary endothelium by electron microscopy. We studied the pharmacological effect of ICAM-directed targeting of tissue-type plasminogen activator (tPA). Anti-ICAM/tPA, but not control IgG/tPA, conjugate accumulates in the rat lungs, where it exerts plasminogen activator activity and dissolves fibrin microemboli. Therefore, ICAM may serve as a target for drug delivery to endothelium, e.g., for pulmonary thromboprophylaxis. Enhanced drug delivery to sites of inflammation and the potential anti-inflammatory effect of blocking ICAM-1 may enhance the benefit of this targeting strategy.

INTRODUCTION

Thrombosis and inflammation are often intertwined processes that contribute to cardiovascular morbidity and mortality. In many cases, the pulmonary vasculature is the major site of vascular inflammation and thrombosis and considerable efforts have been expended to develop strategies to target drugs to this site. Yet current methods to manage inflammation-related thrombosis remain sub-optimal [1-3]. For example, targeting fibrin and activated platelets promotes the delivery of anti-thrombotic agents to existing blood clots, e.g. in coronary vessels [4,5]. However, targeting components of pre-formed clots has afforded only modest improvements in experimental models, likely due to limited penetration[6], and such clot-targeting strategies are unlikely to be useful for thromboprophylaxis.

Targeted delivery of anti-thrombotic drugs to the vascular lumen, including those involved by inflammation, prior to clot formation may permit thromboprophylaxis in patients with high propensity for thrombosis. Theoretically, overexpression of certain anti-thrombotic proteins by vascular endothelial cells themselves would help to achieve this goal [7-9]. However, gene therapies are not currently suitable to manage acute conditions [10].

Immunotargeting of therapeutic proteins may provide a complementary strategy suitable for more immediate interventions. Antibodies to diverse determinants are being explored as affinity carriers for drug targeting to endothelium [11-15]. A poorly internalizable, high-density and stably exposed determinant on the endothelial surface, up-regulated and functionally involved in vascular thrombosis and inflammation would provide an ideal target for anti-thrombotic proteins. Previous data indirectly suggest that Inter-Cellular Adhesion Molecule-1 (ICAM) may provide such a target to deliver anti-

inflammatory and, perhaps, anti-thrombotic agents [16-20]. However, neither the endothelial internalization of ICAM antibodies (anti-ICAM), nor the tissue distribution, localization, activity and effects of ICAM-targeted therapeutics have been characterized.

In this work we studied ICAM-directed immunotargeting to endothelium in cell cultures, perfused lungs and animals and found that: i) anti-ICAM is not internalized efficiently by the endothelium; ii) cytokine up-regulation of ICAM expression augments surface targeting, but not internalization, of anti-ICAM; iii) anti-ICAM can be used to produce either internalizable (100-200 nm diameter) or non-internalizable (~1 μm) conjugates; and, iv) ICAM targeting delivers active tPA to the pulmonary vascular lumen and facilitates intravascular fibrinolysis.

MATERIALS AND METHODS

The materials used: Na^{125}I and Na^{131}I from Perkin-Elmer (Boston, MA), iodogen, streptavidin (SA) and 6-biotinylaminocaproic acid N-hydroxysuccinimide ester from Pierce (Rockford, IL), human recombinant tPA from Genentech (San Francisco, CA), β-galactosidase-streptavidin conjugate (SA-β-Gal) from Sigma (St. Louis, MO), chromogenic tPA substrate, SPECTROZYME®tPA, kind gift by American Diagnostica (Greenwich, CT), fluorescein-labeled transferrin or SA from Molecular Probes (Eugene, OR). Monoclonal (IgG) antibodies: mAb R6.5 [21], mAb 1A29 [22] and mAb YN1 [23] against human, rat and murine ICAM-1; mAb 9B9 against human and rat ACE [24], mAb 1009 and mAb 311 against human and murine thrombomodulin, TM [11]. Antibodies against IgG (fluorescent-labeled or gold-conjugated) were from Jackson Immunoresearch (West Grove, PA) and Amersham (Piscataway, NJ).

Conjugation and size determination. Antibodies, IgG and tissue type plasminogen activator (tPA) were biotinylated and radiolabeled using Iodogen without loss of activity, as described [25]. The number of biotin residues per molecule of protein was determined by Immunopure-HABA® assay (Pierce, Rockford, IL) as per manufacturer instructions. Biotinylated tPA or β-Gal was coupled to biotinylated antibodies using streptavidin cross-linking following a three-step procedure described in detail previously [25-27]. The size of the resulting conjugates was determined by Dynamic Light Scattering, as described [25-27]. The conjugates are designated hereafter as anti-ICAM/β-Gal and IgG/β-Gal or anti-ICAM/tPA and IgG/tPA.

Cell culture experiments. Surface binding and intracellular uptake of ^{125}I-labeled antibodies were measured in cultures of human umbilical vein endothelial cells (HUVEC, from Clonetics) and a human mesothelioma cell line expressing ICAM-1 (REN cells) as

described previously for anti-PECAM [28]. Control and TNFα-challenged cells were incubated for 1 hr at either 4°C or 37°C with ^{125}I-anti-ICAM, ^{125}I-anti-ACE, ^{125}I-anti-TM or ^{125}I-control IgG. After washing, surface-bound antibodies were eluted with glycine, while internalized antibodies were measured in the cell lysates.

Suspended control or TNFα-treated HUVEC and REN cells were incubated with 30μg/ml anti-ICAM or anti-TM for 1hr at 4°C, washed, counterstained with FITC-labeled goat anti-mouse IgG (30 min at 4°C), re-suspended in PBS and analyzed by FACS.

Cellular localization of anti-ICAM was visualized using immunofluorescence at 60X magnification. TNFα-treated cells were incubated with 10 μg/ml anti-ICAM for 1hr at either 4°C or 37°C in 1% BSA-containing medium. After washing and fixation, the surface-associated anti-ICAM was stained with Texas Red labeled goat anti-mouse IgG. Thereafter, internalized anti-ICAM was counterstained in permeabilized cells using FITC-labeled goat anti-mouse IgG. Fluorescein-labeled transferrin was used as a control for internalizable ligand in parallel wells. In separate experiments, Rat Pulmonary Microvascular Endothelial Cells (RPMVEC) were incubated with anti-ICAM/SA-Texas Red conjugates of different sizes (100-200 nm or ~1 μm) for 1 hr at 37°C. After cell fixation, surface bound conjugates were counterstained with a FITC-labeled goat anti-mouse IgG.

Experiments in perfused rat lungs (IPL). Lungs were isolated from anesthetized male 170-200 g Sprague-Dawley rats following protocols approved by the Univ. PA. IACUC and were ventilated and perfused for 1 hr at 37°C or 4°C with KRB-BSA buffer containing ^{125}I-labeled antibodies (1 μg unless indicated otherwise), followed by non-recirculating perfusion with KRB-BSA, as described [29,30]. In separate experiments, 100 μg of non-radiolabeled, biotinylated anti-ICAM or anti-ACE was perfused for 1 hr at

37°C. ^{125}I-labeled streptavidin (^{125}I-SA) was added to the perfusate immediately after washing the unbound antibody or after an additional 60 min non-recycling perfusion to measure surface-accessible b-anti-ICAM.

^{125}I-anti-ICAM biodistribution in rats. Anesthetized rats and mice were sacrificed 1 hr after a tail vein injection of a mixture of ^{125}I-anti-ICAM and ^{131}I-IgG (10 μg each) and the radioactivity in blood and major organs (washed with saline, blotted dry and weighed) was measured to calculate the parameters of targeting: percent of injected dose per organ (%ID) or per gram (%ID/g), organ-to-blood ratio (Localization ratio, LR) and immunospecificity index (ISI) (see [29,31] for details).

Biodistribution and tissue localization of anti-ICAM and the anti-ICAM/β-Gal conjugates in animals. The tissue localization of enzymatically active anti-ICAM/β-Gal 1 hr after the tail vein injection of 100 μg conjugate in BALB/c mice was visualized by histological analysis of X-Gal staining in the tissues, as described previously for anti-PECAM/β-Gal conjugate [26]. In a similar experiment, lungs were processed for electron microscopy and developed using a gold-conjugated secondary antibody, as described [26].

Characterization of tPA activity in lung tissue. Anti-ICAM/tPA conjugate or control preparations (IgG/tPA and tPA) were perfused in IPL for 1 hr; unbound materials were eliminated by a 5 min non-recycling perfusion. In one series, lungs were perfused with 3 μg of ^{125}I-labeled tPA conjugated to either anti-ICAM or IgG and the radioactivity was measured. In the next series, aliquots of lung homogenates obtained after perfusion of 100 μg of an unlabeled tPA conjugates were added to ^{125}I-labeled fibrin clots, formed as described previously [30], and the release of ^{125}Iodine into the supernatant at 37°C was measured. In the next series, 750 μl of a 0.4 mmol/l solution of a chromogenic tPA substrate was infused into the lung via the pulmonary artery for 20 min and the optical

density at 405 nm in the outflow perfusate was measured. In the last series, a suspension of ^{125}I-labeled fibrin microemboli (^{125}I-ME, 3-10 micron diameter) prepared as described previously [32] was infused into the common pulmonary artery after perfusion with either anti-ICAM/tPA or IgG/tPA. ^{125}I-ME lodge and degrade slowly in intact isolated perfused rat lungs [30]. The lungs were perfused for 1 hour with buffer containing 20% plasma as a source of plasminogen and the residual radioactivity in the lungs was measured.

Statistics. A t-test or a one-way analysis of variance (ANOVA) (SigmaStat 2.0) were used to determine statistically significant differences ($p < 0.05$) between groups. Post-hoc testing was performed with Fischer Least Square difference test. Data is shown as mean $\pm$ SEM unless otherwise stated.

RESULTS

Endothelial cells internalize anti-ICAM inefficiently. ^{125}I-anti-ICAM, but not

control IgG, bound specifically to unstimulated endothelial cells (HUVEC) (Fig. 1A).

Eighty-85% of the ^{125}I-anti-ICAM bound to cells at 37°C was eluted by glycine one hour

later compared with 90-95% bound at 4°C (not shown). Consistent with this, ~10% of

bound anti-ICAM was internalized by 60 min at 37°C *vs* ~60% of ^{125}I-anti-ACE and [125]I-

anti-TM (Fig. 1B). Therefore, resting endothelial cells internalize anti-ICAM

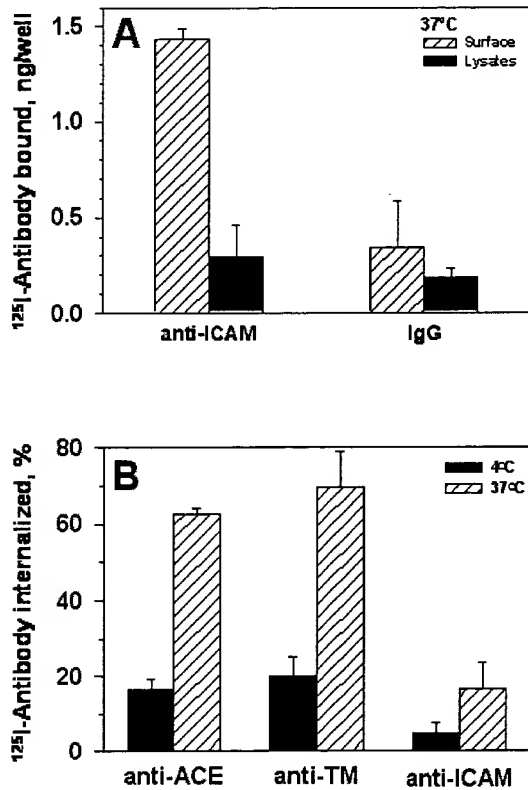inefficiently, leaving ~90% of the antibody on the cell surface.

Figure 1. Resting endothelial cells bind, but do not internalize ^{125}I-anti-ICAM. (A) HUVEC

were incubated with ^{125}I-anti-ICAM or ^{125}I-IgG (1 hr, 37°C) and radioactivity was determined in the surface

fraction (glycine elution) and in the cell lysates. (B) Percent of internalization of ^{125}I-labeled antibodies

against ACE, TM and ICAM-1 by HUVEC at either 4°C or 37°C. The data are expressed as M±SD (n=3).

Anti-ICAM uptake in the perfused rat lungs (IPL). We then asked whether anti-ICAM was handled similarly by intact vascular endothelium under flow. Rat IPL were perfused with ^{125}I-anti-ICAM or control ^{125}I-IgG in a blood-free buffer. ^{125}I-anti-ICAM bound specifically to the lungs (Fig. 2A), reaching saturation at ~10 μg/gram of tissue. Scatchard analysis (inset) revealed that rat lungs contain ~5×10^{13} anti-ICAM binding sites per gram (~1.5-2.5 x 10^5 binding sites per endothelial cell).

We then examined the internalization of anti-ICAM and anti-ACE in IPL. Pulmonary uptake of ^{125}I-anti-ACE was markedly lower at 6°C than at 37°C, likely due to inhibition of the energy-dependent uptake of antibody. In contrast, practically the same uptake of ^{125}I-anti-ICAM was seen at 6°C and at 37°C (Fig. 2B). This result reflects minor, if any, contribution of an energy-dependent internalization pathway for anti-ICAM in IPL.

The IPL setting permits sequential perfusion of ^{125}I-SA immediately or 1 hr after biotinylated antibodies, to test the accessibility of endothelium-bound antibodies to the circulation. Binding of ^{125}I-SA in the lungs was reduced by 70% when biotinylated anti-ACE was allowed to remain in the vasculature for 1 hr at 37°C, indicating that antibody disappearance from the lumen. In contrast, binding of ^{125}I-SA after perfusion of biotinylated anti-ICAM did not diminish with time, indicating that the endothelium-bound anti-ICAM remains accessible from the lumen at 37°C (Fig. 2C). Therefore, pulmonary endothelium under flow conditions avidly binds, but internalizes anti-ICAM poorly.
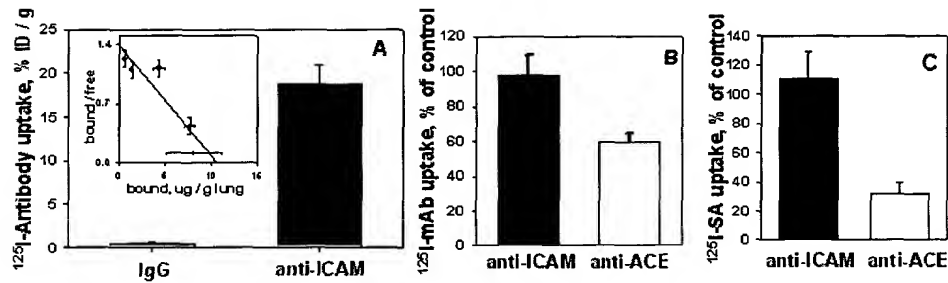
Figure 2. ^{125}**I-anti-ICAM accumulates without internalization in the isolated rat lungs.** (A) Accumulation of ^{125}I-anti-ICAM or ^{125}I-IgG perfused for 1 hr at 37°C. Inset shows a Scatchard analysis of ^{125}I-anti-ICAM binding. (B) Temperature dependence of anti-ICAM uptake (closed bars) and anti-ACE uptake (hatched bars) in the lungs. At 4°C, the pulmonary uptake of ^{125}I-anti-ACE is inhibited, whereas the uptake of ^{125}I-anti-ICAM is not affected (uptake at 4°C is shown as percent of the 100% control value attained at 37°C). (C) Disappearance of anti-ICAM (closed bars) and anti-ACE (hatched bars) from the luminal surface in the lungs perfused at 37°C. After accumulation in the lungs, biotin-anti-ICAM, but not biotin-anti-ACE, is accessible to the blood for a prolonged time. ^{125}I-streptavidin (^{125}I-SA) was perfused in the lungs either immediately after biotinylated antibodies accumulation or after 60 min additional perfusion at 37°C with antibody-free buffer. Data of ^{125}I-SA uptake post 60 min delay are shown as percent of that observed immediately after biotinylated antibodies accumulation (100% level).

TNFα stimulates anti-ICAM binding, but not internalization. TNFα augmented anti-ICAM binding to HUVEC, but inhibited anti-TM binding, while REN cells, that do not express thrombomodulin, bound anti-ICAM constitutively at a relatively high level that was augmented further by TNFα (FACS analysis, Fig. 3A).

FACS results were confirmed by ^{125}Iodine tracing studies using HUVEC monolayers. TNFα markedly augmented binding of ^{125}I-anti-ICAM, but not control ^{125}I-IgG (Fig. 3B). However, the intracellular uptake of ^{125}I-anti-ICAM by TNFα-stimulated cells after a 1 hr incubation at 37°C was equivalent in HUVEC (11.6±0.7%) and REN cells (10.1±2.7%); background levels at 4°C were 5.1±0.8 and 3.2±0.6%, respectively.

These radiotracer data showing minimal (no more than 10%) internalization of anti-ICAM by cytokine-stimulated cells were confirmed by immunofluorescence microscopy. Figure 3C shows typical images of TNFα-stimulated HUVEC and REN cells incubated with anti-ICAM for 1 hr at either 4°C or 37°C and stained before or after permeabilization with Texas Red and FITC-labeled secondary antibody. The staining of intact and permeabilized cells were essentially identical, showing predominantly dual (yellow) labeling of the surface-bound anti-ICAM both at 4°C and 37°C, with no appreciable green staining (representing internalized anti-ICAM). As a control, the intracellular staining of HUVEC incubated with internalizable fluorescein-labeled transferrin was evident at 37°C, but not at 4°C (Panels 1 and k). Therefore, TNFα markedly up-regulates anti-ICAM binding to endothelial and mesothelial cells, but does not augment anti-ICAM internalization.
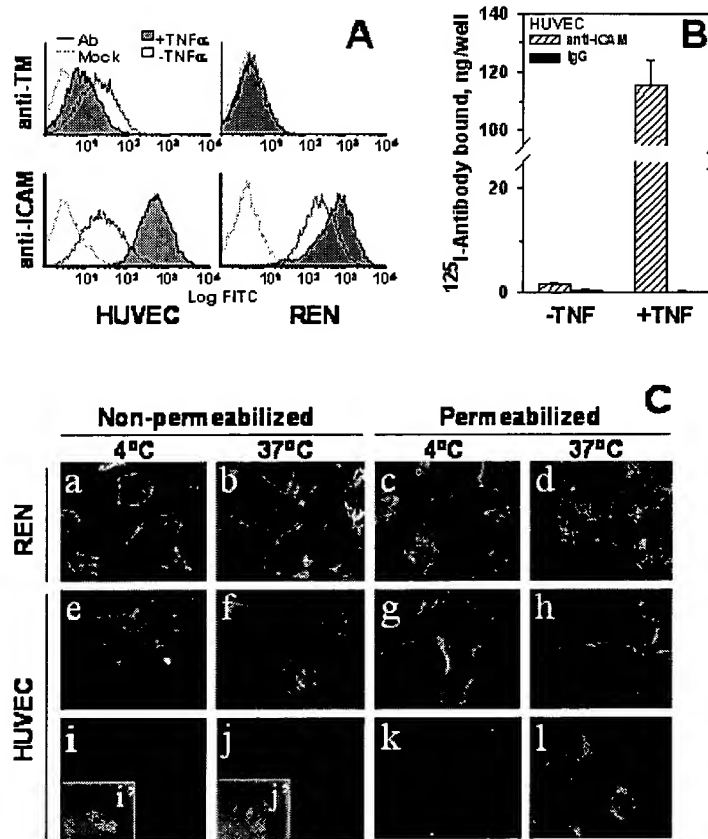
Figure 3. TNFα increases anti-ICAM binding, but not internalization by endothelial and mesothelioma cells. (A) FACS analysis using anti-ICAM and anti-TM. TNFα suppresses expression of thrombomodulin (TM, upper panels) by HUVEC and stimulates that of ICAM-1 (lower panels) by HUVEC and REN cells. Dashed line: antibody-free medium; resting (☐) or TNFα challenged (■) cells. (B) ^{125}I-anti-ICAM binding to resting and TNFα-treated HUVEC monolayer. The data is shown as M±SD, n=4. Panel C. Fluorescent micrographs (60X) of TNFα-stimulated cells incubated with anti-ICAM. The cells were incubated at 4°C or 37°C with anti-ICAM (panels a-h), antibody-free medium (i, j) or transferrin (k, l). After washing and fixation the cells were sequentially stained with Texas Red secondary antibody, permeabilized and counterstained with FITC-labeled secondary antibody (yellow, surface-bound anti-ICAM; green, internalized anti-ICAM). On the left, the non-permeabilized cells were stained with both Texas Red and FITC-labeled antibodies (positive control for surface staining, yellow color). Green color corresponds to the intracellular staining (see panels k and l showing staining of HUVEC incubated with fluorescein-labeled transferrin). Insets i' and j' show phase contrast images in controls.

Biodistribution of radiolabeled anti-ICAM in vivo. ^{125}I-anti-ICAM, but not ^{131}I-

IgG, accumulated in the lungs after intravenous injection in rats (Fig. 4) and in mice (not

shown). Significant uptake was also seen in the liver and spleen, but anti-ICAM uptake

per gram of tissue was always greatest in the lungs. Pulmonary uptake of anti-ICAM was

20-30% lower after intra-arterial injection (not shown).

We analyzed the specificity of anti-ICAM targeting to pulmonary tissue. In rats,

the pulmonary uptake of ^{125}I-anti-ICAM was ~17% ID/g (Fig. 4A), an immunospecificity

index (ISI$_{\%ID/g}$, ratio of %ID/g of anti-ICAM to that of IgG) of ~25, ten-fold higher than

that in the liver and spleen (Fig. 4B). The blood level of anti-ICAM was lower than that

of control IgG, likely due to depletion of the circulating pool. The anti-ICAM pulmonary

Localization Ratio (LR, tissue-to-blood ratio) was ~50 (Fig. 4C), while the IgG LR was

~0.2. Therefore, the pulmonary ISI$_{LR}$ calculated using anti-ICAM and IgG LR, thereby

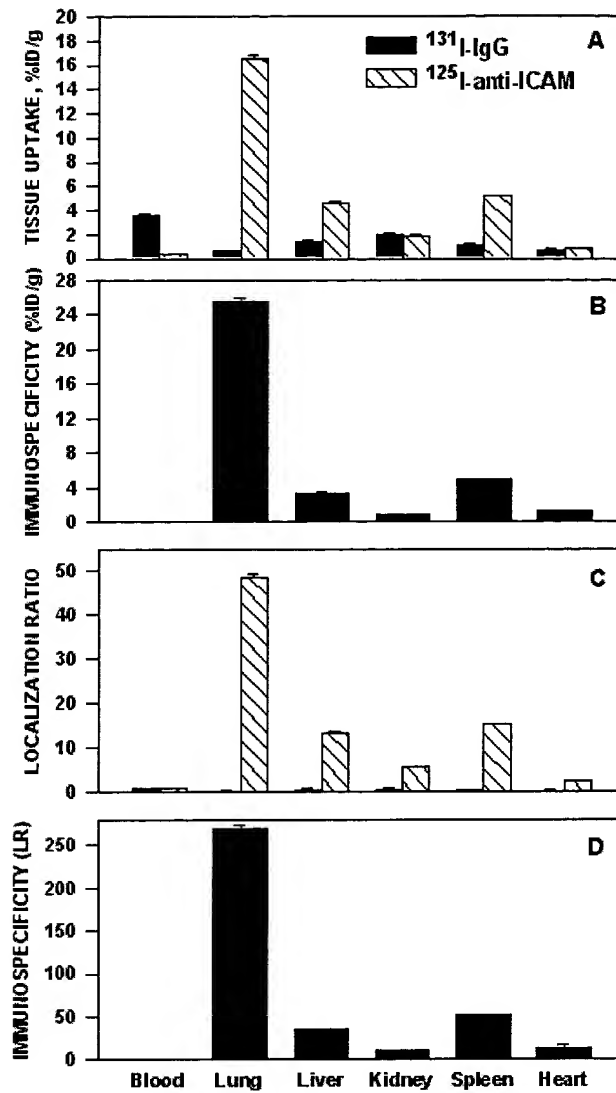correcting for the blood level, was ~250 (Fig. 4D).

Figure 4. Pulmonary targeting of [125]**I-anti-ICAM in rats.** Biodistribution of ^{125}I-anti-ICAM

(hatched bars) or ^{131}I-IgG (closed bars) 1 hr after intravenous injection in anesthetized rats. The data is

shown as M±SEM, n=4. (A) Absolute values of the uptake in organs expressed as percent of injected dose

per gram. (B) Immunospecificity index (ISI$_{\%ID/g}$), calculated as ratio of anti-ICAM to IgG %ID/g. (C)

Localization Ratio, LR, calculated as ratio of %ID/g in an organ to that in blood. (D) ISI$_{LR}$ calculated as

ratio of anti-ICAM LR to IgG LR.

Effects of pro-inflammatory challenges on anti-ICAM targeting. Endotoxin facilitated pulmonary uptake of ^{125}I-anti-ICAM, likely due to up-regulation of endothelial ICAM in response to cytokines. In rats, LPS caused a 30% increase in the pulmonary uptake of ^{125}I-anti-ICAM (Fig. 5), with a concomitant reduction in the blood level (pulmonary LR almost doubled from 50 to 85). In contrast, LPS suppressed pulmonary uptake of ^{125}I-anti-ACE in rats by 50% (pulmonary LR reduced from 14 to 7). Therefore, anti-ICAM targeting in LPS-treated rats was ten times more robust than that of anti-ACE (LR 85 vs 7). Pulmonary targeting of ^{125}I-anti-ICAM was stably enhanced at 5 and 24 hr post LPS injection (LR 77 and 85). A similar elevation of ^{125}I-anti-ICAM pulmonary targeting was seen in LPS-treated mice (not shown). The ^{125}I-anti-ICAM pulmonary uptake was doubled in mice exposed to 98% O_2 atmosphere, in contrast with a 50% decrease in the ^{125}I-anti-TM uptake (not shown). Therefore, pro-inflammatory factors suppress anti-ACE and anti-TM, but augment anti-ICAM targeting.
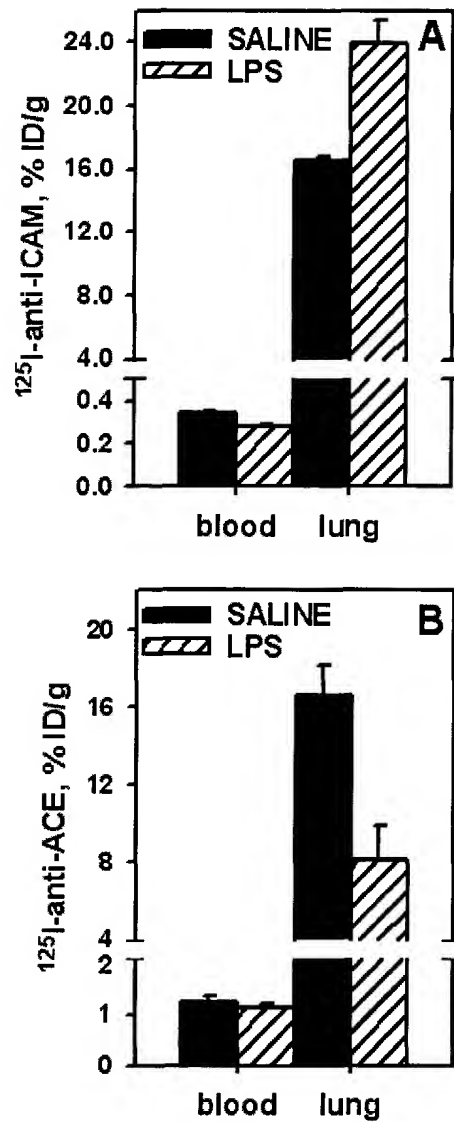
Figure 5. Endotoxin enhances [125]**I-anti-ICAM pulmonary targeting.** ^{125}I-anti-ICAM (left panel) or ^{125}I-anti-ACE (right panel) was injected in control rats (hatched bars) or after intraperitoneal injection of LPS (closed bars). Lung and blood level of ^{125}I was determined 1 hr later.

Visualization of ICAM-directed vascular immunotargeting in animals. We visualized the pulmonary localization of anti-ICAM in mice by electron microscopy. Specific binding of the secondary gold-labeled antibody was evident in lungs harvested 4 hr after anti-ICAM injection (Fig. 6A). Semi-quantitative analysis after anti-ICAM

injection revealed 16 ± 3 endothelium-associated particles/field vs 3 ± 1 particles/field associated with alveolar epithelium and interstitium ($M\pm SEM$, ten fields). Anti-ICAM was primarily localized along the luminal surface of the endothelium (arrows in Panel a). Noteworthy, we did not see endocytic vacuoles containing gold particles, the hallmark of endothelial uptake of internalizable conjugates in the lungs [26].

To test whether anti-ICAM delivers an active enzyme cargo to endothelium, we conjugated a reporter enzyme, β-Galactosidase with anti-ICAM or control IgG. Figure 6B shows the results of X-Gal staining of the organs 1 hr post injection of either anti-ICAM/β-Gal or IgG/β-Gal conjugate in mice. After IgG/β-Gal injection (Panels e-h), β-Gal activity was seen in the peripheral zone of the splenic follicles, the known site of Fc-receptor mediated uptake of immunoconjugates [26,33]. The splenic follicles were also stained by anti-ICAM/β-Gal (Fig. 6B, Panel a) as were the renal glomeruli (Fig. 6B, Panel b), the known site of β-Gal elimination [26,33].

However, injection of anti-ICAM/β-Gal, but not IgG/β-Gal, delivered β-Gal activity to the lungs (compare Panels d and h). Anti-ICAM/β-Gal was concentrated in the alveolar capillaries and in the lumen of larger vessels; no β-Gal activity was seen in sub-endothelial layers of blood vessels, interstitium or airways (Fig. 6B, Panels i and j).
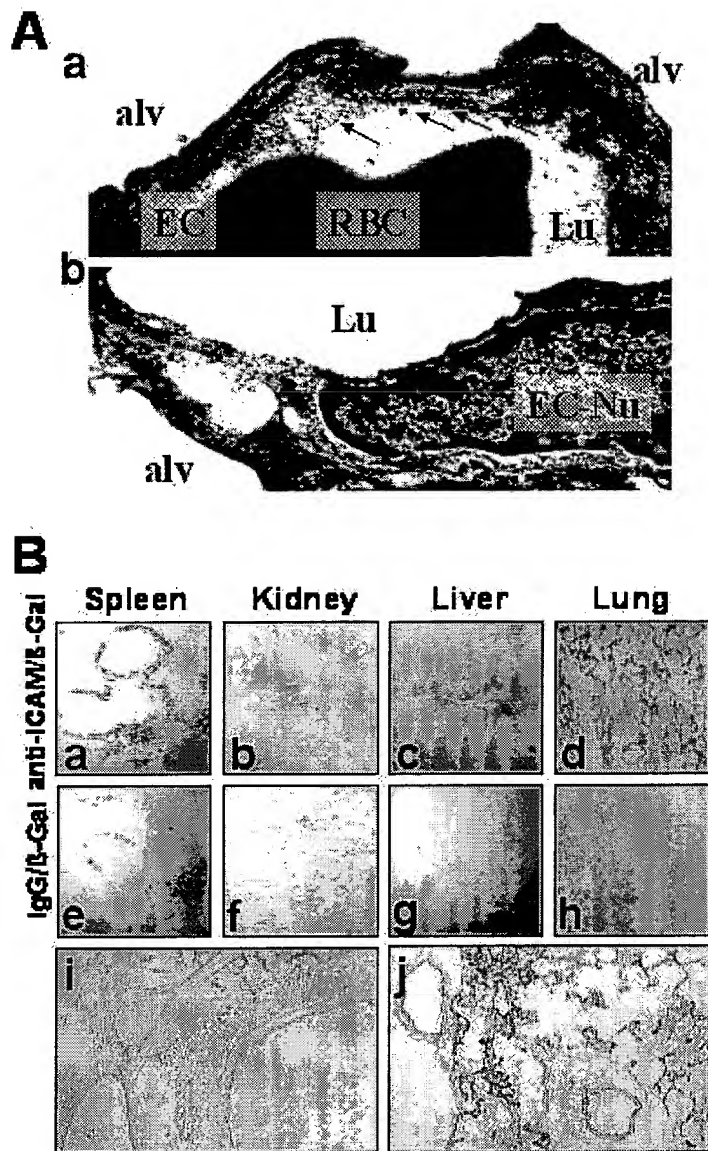
Figure 6. Localization of anti-ICAM and anti-ICAM/β-Gal in the pulmonary vasculature after injection in mice. (A) Immuno-gold electron microscopy of the lungs harvested 4 hr after intravenous injection of 100 μg of anti-ICAM (upper panel, a) or control IgG (lower panel, b). Arrows show endothelium-associated gold particles. RBC, red blood cell in a capillary lumen; Lu, vascular lumen; Alv, alveolar compartment, EC, endothelial cell. (B) Targeting of an active β-Gal conjugates was visualized 1 hr post injection in mice, using standard X-Gal staining protocol. Distribution of anti-ICAM//β-Gal (panels a-d) and control IgG//β-Gal (panels e-f). Detailed view of anti-ICAM//β-Gal in the lungs (panels i and j).

Effect of anti-ICAM conjugates size on endothelial internalization. Streptavidin

was cross-linked to biotinylated anti-ICAM and other biotinylated proteins (e.g., b-tPA,

see below) at varying molar ratios of reactants [25-27]. Analysis using dynamic light

scattering analysis showed that conjugates ranging in size from 100 nm to several

microns were generated depending on the molar ratio between SA and biotinylated anti-

ICAM (Figure 7A). Double staining of fluorescent-labeled anti-ICAM conjugates

revealed that rat microvascular endothelial cells internalized those anti-ICAM conjugates

having a diameter of 100-200 nm, but did not internalize large conjugates around 1 μm

(Fig. 7B). In fact, after a 1 hr incubation at 37°C, the large, 1-2 micron double-labeled

anti-ICAM conjugates (the preparation that corresponds to the peak farthest to the right in

Fig. 7A) decorated the entire cell surface (Fig. 7C). Control IgG conjugates did not bind

to endothelium irrespective of size and did not accumulate in the isolated rat lungs (not
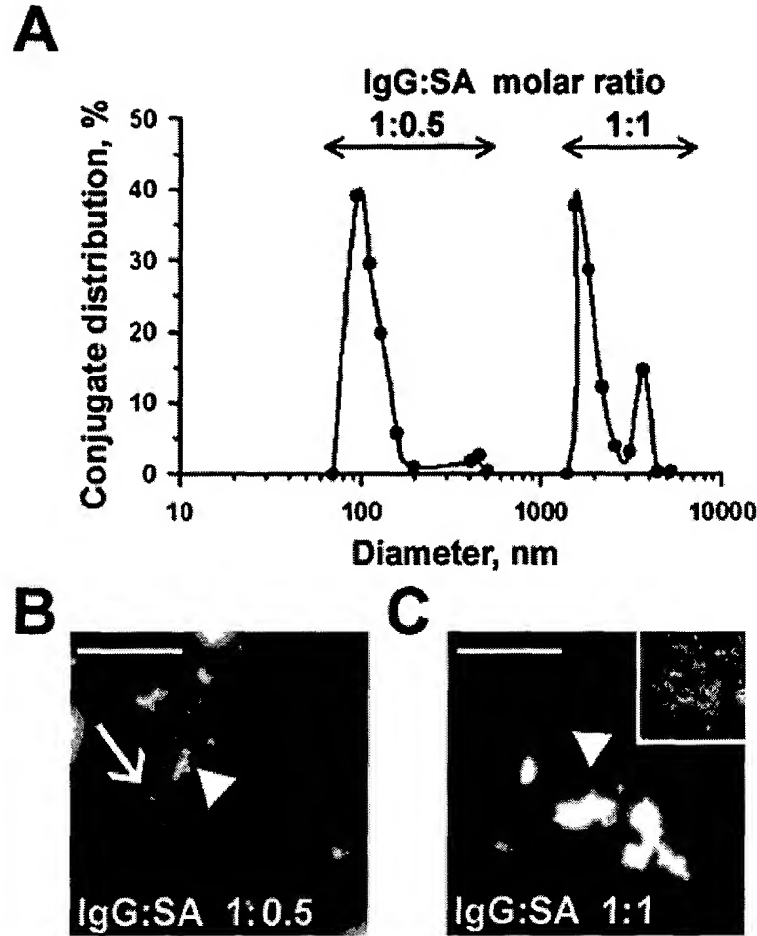
shown).

Figure 7. Size of anti-ICAM conjugates modifies their uptake by endothelial cells. A. DLS

analysis of size distribution of the conjugates prepared at molar ratio between biotinylated anti-ICAM and

streptavidin of 1:0.5 (left peak) or 1:1 (right peak). B. RPMVEC were incubated for 1 hr at 37°C with anti-

ICAM conjugates containing rhodamine-labeled streptavidin with mean diameters of 100-200 nm (panel B)

or larger than 1 μm (panel C). The surface-bound fraction of the conjugate was double-labeled using a

FITC-labeled secondary antibody. Red color (arrows) denotes internalized conjugates, yellow color

(arrowheads) denotes the non-internalizable, larger conjugates. White bars in panels B and C correspond to

5 μm size.

ICAM-directed targeting of tPA to the pulmonary vasculature. We then tested whether anti-ICAM would target an anti-thrombotic drug to sites of inflammation susceptible to thrombosis. To do so, we prepared large (~1 μm), poorly internalizable anti-ICAM/^{125}I-tPA and IgG/^{125}I-tPA conjugates. One hr post intravenous injection, 7% of the injected anti-ICAM/^{125}I-tPA had accumulated in the rat lungs compared with <0.3% for IgG/^{125}I-tPA (Fig. 8A), a pulmonary ISI$_{\%ID/g}$ of 25 (Fig. 8B). The blood level of anti-ICAM/^{125}I-tPA showed a corresponding decrease compared to IgG/^{125}I-tPA. Therefore, the pulmonary LR of anti-ICAM/^{125}I-tPA exceeded 20 (Fig. 8C) with a calculated ISI$_{LR}$ of ~80 (Fig. 8D). Anti-ICAM/^{125}I-tPA was retained in the lungs for at least several hr post injection (not shown). Similar results were obtained in mice (not shown).
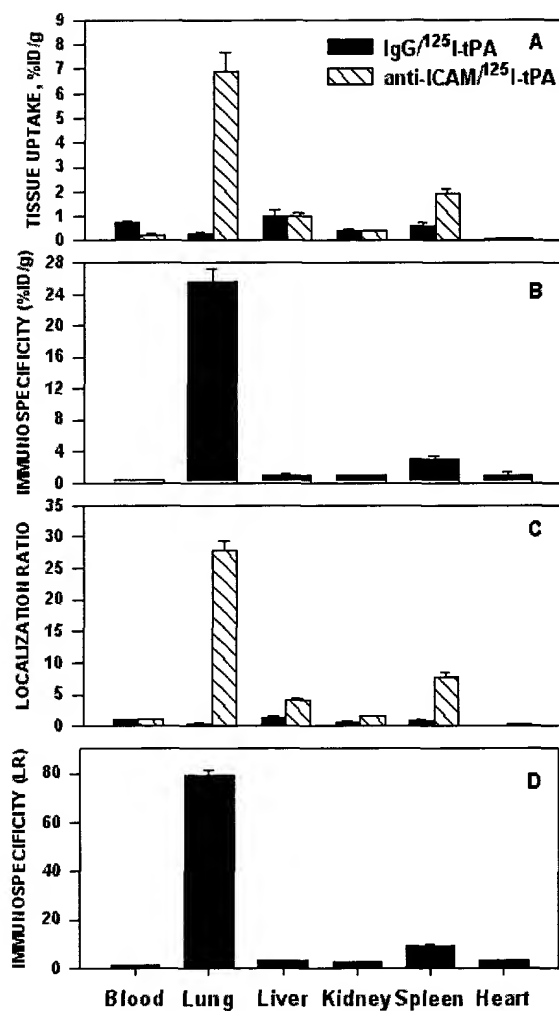
Figure 8. Pulmonary targeting of ^{125}I-tPA conjugated with anti-ICAM in rats. Biodistribution of ^{125}I-tPA conjugated to anti-ICAM IgG (hatched bars) or control IgG (closed bars) 1 hr after intravenous injection in anesthetized rats. The data is shown as M±SEM, n=4. (A) Absolute values of the uptake in organs expressed as percent of injected dose per gram. (B) Immunospecificity index (ISI$_{\%ID/g}$), calculated as ratio of anti-ICAM to IgG %ID/g. (C) Localization Ratio, LR, calculated as ratio of %ID/g in an organ to that in blood. (D) ISI$_{LR}$ calculated as ratio of anti-ICAM LR to IgG LR.

Anti-ICAM/tPA bound to the pulmonary endothelium surface retains plasminogen activity and dissolves intravascular clots. To test whether anti-ICAM can be used to deliver enzymatically active tPA to the endothelial lumen, we perfused anti-ICAM/tPA or IgG/tPA in IPL. In all experiments, the vasculature was washed free of unbound conjugates prior to measuring tPA uptake and activity. Anti-ICAM, but not the control IgG carriage, led to pulmonary accumulation of ^{125}I-tPA (Fig. 9A).

Aliquots of lung homogenates obtained after perfusion of anti-ICAM/tPA or IgG/tPA were then incubated with ^{125}I-fibrin clots at 37°C *in vitro*. Homogenates of lungs perfused with anti-ICAM/tPA caused 10-fold more fibrinolysis, measured by release of ^{125}I, than lungs perfused with IgG/tPA (Fig. 9B).

We then infused a chromogenic tPA substrate into IPL. Enzymatic conversion of the substrate leading to appearance of a colored product was detected in the perfusate outflow of lungs pre-perfused with anti-ICAM/tPA, but not those pre-perfused with IgG/tPA (Fig. 9C). Therefore, the anti-ICAM/tPA associated with the luminal surface of the pulmonary endothelium retains its enzymatic activity.

Accessibility to a small synthetic substrate (m.w. <500 D) does not prove that the anti-ICAM/tPA is accessible to convert its protein substrate, plasminogen. To examine this issue, a suspension of ^{125}I-microemboli was infused into the pulmonary artery 1 hr after perfusion with either anti-ICAM/tPA or IgG/tPA. The radioactivity in the lungs was determined 1 hr later as a measure of residual unlysed fibrin clots. Lungs pre-perfused with anti-ICAM/tPA practically completely dissolved the radiolabeled fibrin clots, whereas fibrinolysis in the lungs perfused with IgG/tPA did not differ significantly from the basal level measured in control lungs (Fig. 9D). Therefore, anti-ICAM/tPA

accumulates in the lungs, resides in enzymatically active form on the luminal endothelial

surface, and thereby markedly facilitates fibrinolysis in the pulmonary vasculature.
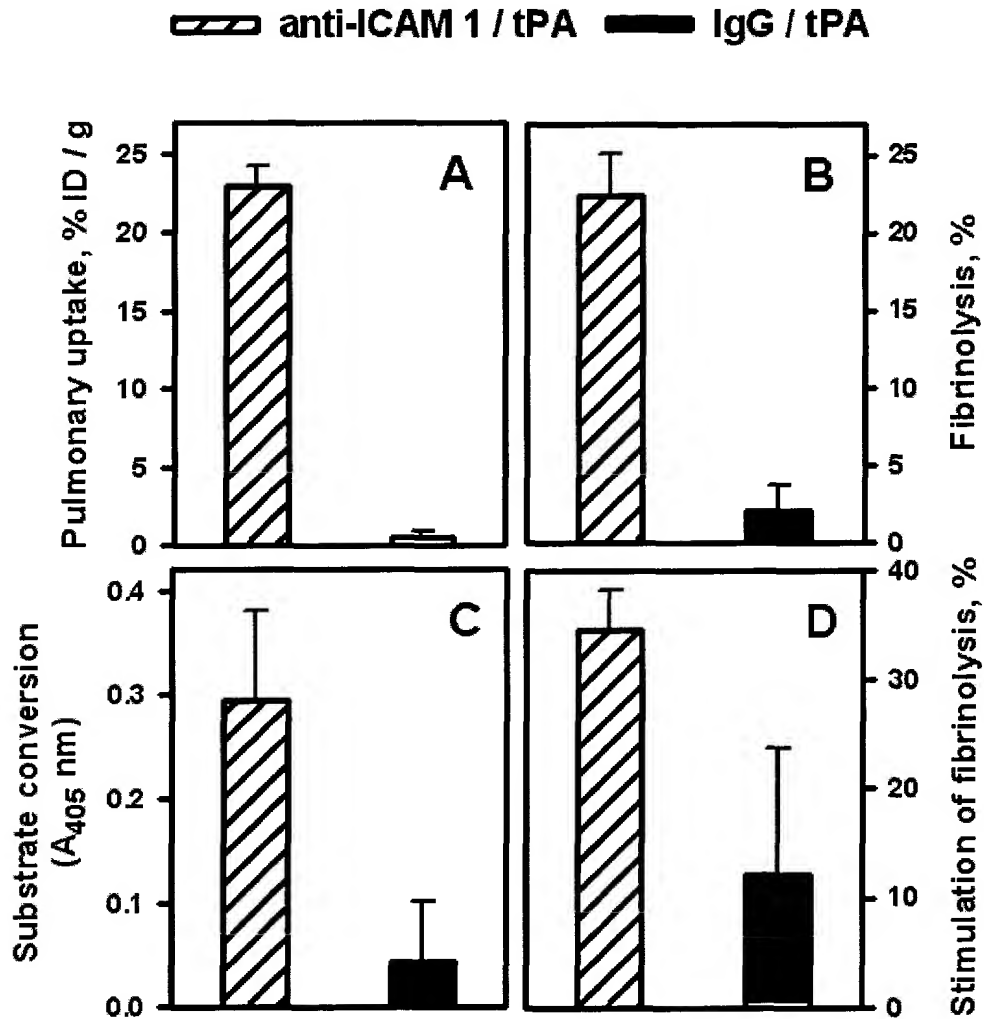

Figure 9. Anti-ICAM/tPA accumulated in pulmonary vasculature facilitates fibrinolysis.

Isolated rat lungs were perfused with anti-ICAM/tPA (hatched bars) or IgG/tPA (closed bars) for 30 min at

37°C and washed free of unbound conjugates (5 min non-circulating perfusion with a buffer). (A)

Pulmonary uptake of ^{125}I-tPA conjugated to either anti-ICAM or control IgG. (B) Fibrinolysis of fibrin

clots by aliquots of lung homogenates obtained after perfusion. (C) Conversion of chromogenic tPA

substrate perfused after the conjugates. (D) Dissolution of radiolabeled fibrin emboli lodged in the

pulmonary vasculature after perfusion of the conjugates. The data shown as M±SEM, n=4.

DISCUSSION

Drug targeting and concurrent blocking of a non-internalized, highly expressed, pro-inflammatory determinant expressed on the endothelial lumen that is stably up-regulated in the perturbed vasculature may provide a specific and powerful approach for treatment and prophylaxis of vascular inflammation and thrombosis. Our data indicate that ICAM-1 (CD54) fulfills the criteria of an "ideal" target for this specific goal and that anti-ICAM may be used for vascular immunotargeting of anti-thrombotic drugs.

Static human endothelial cells cultured under sterile conditions constitutively express relatively modest levels of ICAM-1 (Fig. 1). However, the level of expression is much higher in vivo [34] and anti-ICAM binds to the resting endothelium in intact animals [20,34]. Diverse cell types express ICAM-1, but the largest fraction directly accessible to the bloodstream is exposed on the endothelial surface [34]. This fact explains anti-ICAM targeting in vascularized organs (Fig. 2 and 4) and confinement of the targeted cargoes to the vascular lumen (Fig. 2, 6 and 9).

The pulmonary vasculature is the first major capillary network encountered by intravenously injected antibodies, contains roughly one-third of the endothelium in the body, is exposed to the entire cardiac output of venous blood and, therefore, comprises the preferred target for affinity carriers recognizing pan-endothelial determinants [28,31]. Importantly, pulmonary uptake of anti-ICAM and anti-ICAM conjugates is not due to a non-specific binding or mechanical retention in the vasculature, as control IgG counterparts neither bound to HUVEC, nor accumulated in the lungs. In fact, the immunospecificity of the anti-ICAM and anti-ICAM/tPA conjugate pulmonary accumulation in normal rats approaches values of 250 and 50, respectively (Fig. 4 and 8). Analysis of the quantitative binding data obtained in rat lungs (Fig. 2) indicates that

binding of ~5-50 mg anti-ICAM can be expected in the human pulmonary vasculature. Thus, anti-ICAM carriers are likely to provide robust and preferential targeting to the pulmonary endothelium in intact animals, matching the characteristics of the best candidate carriers tested to date, including antibodies directed against PECAM, ACE and a caveoli-associated antigen gp90 [15,24,28,31,33].

Certain pathological conditions suppress targeting to other constitutive endothelial determinants, such as thrombomodulin and ACE [18,35,36]. For example, endotoxin inhibits anti-ACE targeting in rats by 50% (Fig. 5). In contrast, cytokines, oxidants, abnormal shear stress and thrombin [37] are all known to enhance endothelial ICAM-1 expression [34,38] and augment anti-ICAM vascular targeting *in vivo* [16,20]. Up-regulation of endothelial ICAM-1 expression by thrombin [37] also makes it a preferred candidate for delivering anti-thrombotic agents. Our data extend these observations and reveal that: i) cytokine stimulation does not augment anti-ICAM internalization (Fig. 3) and, ii) pulmonary targeting of anti-ICAM is stably augmented in models of pro-inflammatory challenge *in vivo* (e.g., Fig. 5). This feature distinguishes targeting ICAM from targeting selectins, which are only transiently exposed on the perturbed endothelium [39].

Antibodies and conjugates may unintentionally suppress important functions of endothelial proteins with potentially deleterious consequences (e.g., thrombosis) making them less suitable for the therapeutic targeting [40]. ICAM-1, a counter-receptor for leukocytes integrins, supports cell adhesion on endothelium [34,41]. Since anti-ICAM suppresses inflammation by blocking leukocyte adhesion [34,38,41-44], drug targeting to endothelial ICAM-1 is unlikely to have unintended deleterious effects on the host and, indeed, may provide secondary therapeutic benefits against inflammation, thrombosis and oxidative stress. This feature of anti-ICAM conjugates deserves additional investigation.

Published studies on anti-ICAM internalization have yielded inconsistent results: epithelial and blood cells have been reported to internalize ICAM ligands *in vitro* [45,46], but fragmentary data in other cell types showed the opposite outcome [47,48]. Our studies in cell culture, perfused rat lungs and in animals show that endothelial cells internalize ICAM antibodies poorly (Fig. 1, 2, 3 and 6). Thus, ICAM seems to be well suited for drug targeting to the luminal surface. This feature distinguishes ICAM from other similarly prevalent endothelial determinants all of which are rapidly internalized including thrombomodulin and ACE (Fig. 1 and 2), selectins [47-50] and caveoli-associated antigens [15]. A monoclonal antibody against gp85 antigen accumulates in the rat lungs and is not internalized and therefore can be used for surface targeting in this species [29], but the identity, function and regulation of its human counterpart are not known.

The uptake of anti-ICAM conjugates is modified by their size: endothelium internalizes conjugates with a mean diameter 100-200 nm, but not anti-ICAM conjugates larger than 1μm (Fig. 7). This result indicates that anti-ICAM follows the paradigm observed previously with antibodies directed against another non-internalizable determinant, PECAM-1 [26-28]. Conjugate size can be readily and stably modulated by varying the molar ratios of the reactants, as measured by DLS (Fig.7). In theory, therefore, anti-ICAM represents a carrier that can be modified to facilitate drug delivery to either the endothelial surface (using monomolecular conjugates or conjugates larger than 500 nm) or to the intracellular compartment (using 100-300 nm conjugates). The anti-ICAM/tPA conjugates used in the present study were around 1 μm size, which exceeds the effective internalizable size. Clearly, anti-ICAM/tPA bound along the cell surface retains enzymatic activity in the pulmonary vascular lumen and augments intravascular fibrinolysis (Fig. 9).

This result serves as a proof-of-principle that ICAM represents a suitable target to deliver anti-thrombotic agents to the endothelial lumen. Although anti-ICAM/tPA conjugate itself may represent a useful agent, we anticipate that the strategy can be further optimized by generation of a monomolecular non-internalizable anti-ICAM/tPA fusion protein.

To our knowledge this is the first study showing that fibrinolytic agents can be used in a prophylactic mode to protect vital vascular beds including that in the lungs. Patients are at high risk to develop pulmonary emboli after trauma, when extensive proximal deep venous thrombi are present, after recent pulmonary emboli, in the setting serious respiratory compromise due to diverse cardiopulmonary disease or when the risk of bleeding after systemic anticoagulation is prohibitive [1,2]. Our data suggests that targeted delivery of anti-thrombotic agents to the pulmonary endothelium itself may be a suitable alternative in some of these settings, although additional studies will be needed to establish the duration and extent of fibrinolytic activity that is delivered.

In summary, ICAM possesses a number of highly desirable characteristics as a target for anti-thrombotic and anti-inflammatory drug delivery. Anti-ICAM targeting may allow a spectrum of novel therapeutic approaches, for example a strategy to facilitate the anti-thrombotic potential of the pulmonary vasculature in patients at high risk to develop acute lung injury (ALI/ARDS) and thromboembolism. Future studies in larger animals will define potential therapeutic applicability and limitations of this strategy.

ACKNOWLEDGEMENTS

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